Clinical Research Division
59th Medical Wing, Lackland AFB, Texas

&

Enteric Diseases Department
Armed Forces Research Institute for the Medical Sciences
Bangkok, Thailand

Final Report

AF/SGR AFMSA/SG5I RDT&E FY11 - FY13:

PRE-CLINICAL TESTING OF REAL-TIME PCR ASSAYS FOR DIARRHEAL DISEASE AGENTS OF GENERA *ESCHERICHIA* AND *SHIGELLA*

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14. ABSTRACT

Pre-clinical test results qualify ETEC and Shigella real-time PCR assays as lead candidates for transition to clinical phase testing. Diagnostic sensitivity results were ≥ 96% to ≤ 100% in testing conducted under laboratory and field conditions. Current commercially available molecular-based diagnostic assay sensitivity is ≥ 95% to ≤ 98% representing the standard that must be met or exceeded to qualify as a candidate for FDA clearance. In addition to test activities, Enterotoxigenic Escherichia coli Detection Kit and Shigella Detection Kit pre-IDE documents were prepared to serve as a point of departure for discussion with the FDA Office of In Vitro Diagnostic Device Evaluation and Safety (OIVD) on guidance and clarification of specific testing requirements for eventual clearance.

During the conduct of RDT&E activities a formal GME training program was established by the investigators. The program provides for scholarly and challenging research opportunities in a real-world environment. Under this project, an Air Force resident physician

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Objectives

1. Pre-clinical trial validated ETEC and *Shigella* assays qualified for clinical phase testing

Pre-clinical test results qualify ETEC and Shigella real-time PCR assays as lead candidates for transition to clinical phase testing. Diagnostic sensitivity results were ≥ 96% to ≤ 100% in testing conducted under laboratory and field conditions. Current commercially available molecular-based diagnostic assay sensitivity is ≥ 95% to ≤ 98% representing the standard that must be met or exceeded to qualify as a candidate for FDA clearance. Results are provided in final reports.

In addition to test activities, Enterotoxigenic *Escherichia coli* Detection Kit and *Shigella* Detection Kit pre-IDE documents were prepared to serve as a point of departure for discussion with the FDA Office of In Vitro Diagnostic Device Evaluation and Safety (OIVD) on guidance and clarification of specific testing requirements for eventual clearance.

2. Report describing ABI 7900 and RAPID/JBAIDS pre-clinical test results

Testing was successful (See Results section). Analytical test results are shown in Appendix C.

3. Completed PEC, NEC, IPC and comparator test evaluations

Controls and comparator test are established (See Results section).

4. Quarterly Progress and Expense Analyses Reports and Final report submitted to the Defense Technical Information Center (DTIC)

Copies of reports can be obtained through Project Manager, 59th MDW/ST.

5. Graduate Medical Education (GME) research project completed

During the conduct of RDT&E activities a formal GME training program was established by the investigators. The program provides for scholarly and challenging research opportunities in a real-world environment. Under this project, an Air Force resident physician completed research which directly resulted in advancing Force Health Protection diarrheal disease diagnostic technologies toward clearance. Two separate research projects were completed, abstracts prepared, and posters presented at a medical symposium. The resident successfully completed WHAMC Pathology Department Research Elective 144. These projects are described in the Results section. Project activities are provided in Appendix A and course description and requirements are provided in Appendix B.

Summary

The objectives of this study were accomplished. Real-time diarrheal disease causative agent detection capability was advanced through pre-clinical test phase. The GME component of this study was successfully completed.

The results of this study support qualification of the assays as candidates for FDA clearance as well as for use in environmental (non-human) surveillance. As such, a pre-investigational device exemption (pre-IDE) document was prepared. The pre-IDE document describes the detection technology and its intended use, proposed analytical testing and clinical evaluation strategies. The intent of FDA guidance meetings are to ensure that proposed testing strategy is in line with current OIVDES thinking and is sufficient to support a pre-market notification application. Investigational device exemption (IDE) will all allow use in a clinical study in order to collect safety and effectiveness data required to support a Premarket Approval (PMA) application or a Premarket Notification [510(k)] submission to FDA. Included in FDA OIVDES guidance meetings will be discussion on potential pre-IDE submissions for high throughput systems (HTS) and microarray systems. The above activities will require additional funding under a separate protocol.

This project was funded by the Air Force Medical Support Agency (AFMSA), Research, Development and Innovations Directorate (SG5I), Office of the Surgeon General (AF/SGR) Falls Church, Virginia and the Military Infectious Diseases Research Program (MIDRP), USAMRC, Fort Detrick, Frederick, Maryland. Project activities were conducted by the Enteric Diseases Department, Armed Forces Research Institute for the Medical Sciences (AFRIMS) and Clinical Research Division (CRD)/59th MDW. This project was jointly funded and executed under memorandum of agreement (MOA) between Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland & 59th Medical Wing (MDW) Lackland AFB, Texas (MOA 2007 - 2013. Agreement No.: DODI 4000.19; AFI 25-201).

Products Completed

Point-of-care high throughput system (HTS) and deployable real-time PCR detection capability for diagnosis of etiologic agents of diarrheal disease were accomplished. Pre-clinical test phase demonstrated that ETEC LT, STIa, STIb, and *Shigella* spp. assays exceeded the objective diagnostic sensitivity. The assays proved specific in testing using a broad panel of clinically significant and genotypically similar organisms.

Purpose

The work completed under this project is follow-on to joint projects completed through previous AF/SGR AFMSA/SG5I funded efforts undertaken by the 59th MDW and Department of Enteric Diseases, AFRIMS. Success in these collaborative efforts has positioned military significant disease agent diagnostics for FDA clearance. Under this project, advanced to clinical test phase are real-time PCR assays for enterotoxigenic *Escherichia coli* (ETEC) toxin genes, Heat Stable Ia (STIa), Heat Stable Ib (STIb), and Heat Labile (LT) and *Shigella* enteroinvasive ***E. coli*** (EIEC) spp. The associated training objective provided a scholarly and challenging opportunity in GME.

Pre-clinical testing of previously established ETEC and *Shigella* PCR assays was conducted using two functionally distinct FDA cleared real-time PCR instruments. The first is state of the art laboratory equipment, the Fast Real-Time PCR System (Applied Biosystems, Inc. 7900). This HTS is capable of rapidly screening large numbers of samples, hundreds to thousands, per day. Rapid identification of bacterial disease agents by HTS screening of clinical and environmental samples provides for efficacious treatment and disease prevention. The second PCR instrument is the portable, field-deployable DoD Joint Biological Agent Identification and Diagnostic System (JBAIDS). Disease outbreaks often occur in developing regions and often

coincide with natural or man-made disasters. In situations of underdeveloped or failing health care infrastructure, the JBAIDS provides a valuable aid in disease surveillance and diagnosis. The JBAIDS is deployed in hours and capable of operating independently of conventional laboratory infrastructure.

Assay testing was conducted in compliance with Good Laboratory Practice (GLP) standards established by the FDA specifically for *in vitro* diagnostic device (IVD) clearance. Pre-clinical test results reported here will be used to seek funding for clinical phase testing as well as biosurveillance kit development.

Problem

The ability of military medical personnel to accurately diagnose and recognize diarrheal disease threats in an operational environment is a high priority. The rapid identification of an infectious agent will allow for prompt, appropriate treatment, thereby minimizing morbidity and mortality. Additionally, knowledge about a specific infectious disease threat will allow for the implementation of appropriate prevention and control measures to protect the fighting force.

Results of Pre-clinical Performance

Diagnostic Sensitivity - pre-clinical phase testing showed that JBAIDS TaqMan assays for enterotoxigenic *Escherichia coli* (ETEC) toxin genes, Heat Stable Ia (STIa), Heat Stable Ib (STIb), and Heat Labile (LT) and *Shigella* enteroinvasive ***E. coli*** (EIEC) spp. exceeded the objective diagnostic sensitivity, $\geq 95\%$ to $\leq 98\%$ [Table 1]. Specificity test results reported here, and in previous testing, showed that the assays are specific (Appendix C).

Table 1. Diagnostic sensitivity of ETEC and Shigella PCR assay direct detection from stool

Assay	RAPID Singleplex AFRIMS, Sept 2013 Sensitivity (%)	RAPID Field Test Singleplex Nepal, Mar 2009 Sensitivity (%)	DIG* ETEC Multiplex AFRIMS Sensitivity (%)	Serotyping Shigella AFRIMS Sensitivity (%)
ETEC LT	100% (29/29)	100% (43/43)	69% (20/29)	Not Tested
ETEC STIa	100% (19/19)	100% (21/21)	76% (16/21)	Not Tested
ETEC STIb	100% (29/29)	100% (30/30)	77% (23/30)	Not Tested
Shigella spp	100% (30/30)	100% (30/30)**	Not Tested	97% (29/30)

*DIG: digoxigenin-labeled probe; ** Confirmed as Shigella by sequencing
Cut-off Ct 42

This study was conducted to determine the performance of the assays for relevant specimen types claimed in future labeling. The study protocol provides patient sample inclusion and exclusion criteria, type and number of specimens, directions for use, and statistical analysis information will be used for potential premarket submission. The specimen types (strains) and

total number of samples were based on diarrheal disease epidemiological data. The objective number of samples was a minimum of 30 confirmed positive using the reference method. Pre-clinical studies were conducted at a single facility utilizing a single laboratory. Testing was conducted by experienced and trained personnel at the Department of Enteric Diseases, AFRIMS. This laboratory is the only DoD facility currently conducting diarrheal disease agent clearance activities. The study population included archived nucleic acid extracts from individuals who presented with diarrheal disease. Results were compared using the established reference method (culture). In addition to culture sequencing of amplicon was used for confirmation testing.

Interference Study

Stool presents a relatively complex challenge in sample preparation. Stool harbors an array of PCR interfering substances that must be removed during the nucleic acid extraction process to help assure an efficacious level of diagnostic sensitivity.

A preliminary interference study was successfully completed using clinically relevant conditions. The interferent used was human blood which represents the primary PCR inhibitory substance encountered in stool specimens. The interferent was tested at the potentially "the worst case" concentration (10% w/w) using two strains of organism (ETEC LT and *Shigella*) to assess the potentially inhibitory effects. Interference testing was conducted at LoD and 1000X LoD concentrations of organism to assess inhibitory effects as well as to assess potential for cross-contamination.

There was no significant difference in ETEC LT assay Ct values for stool prepared with spiked-blood and non-spiked samples (below). Testing conducted using the Shigella assay also did not indicate interference. Throughout testing there was no indication of cross-contamination. Study design and JBAIDS screen shots are shown below.

L1: Lysis

T1 : 10^4 LOD ETEC(LT) + L1 (10uL:1.2mL)
T2 : 10 LOD ETEC(LT) + L1 (10uL:1.2mL)
T3 : 10^4 LOD Shi(ipaH) + L1 (10uL:1.2mL)
T4 : 10 LOD Shi(ipaH) + L1 (10uL:1.2mL)
T5:1.2 mL L1 (Negative control)

} + 300ul of 10% stool suspension in DDW

L2 : Added Whole blood in Lysis (20uL:1.2mL)

Stock of L2 Whole blood: Lysis (100 uL:6ml)

T6 : 10^4 LOD ETEC(LT) + L2 (10uL:1.2mL)
T7 : 10 LOD ETEC(LT) + L2 (10uL:1.2mL)
T8 : 10^4 LOD Shi(ipaH) + L2 (10uL:1.2mL)
T9 : 10 LOD Shi(ipaH) + L2 (10uL:1.2mL)

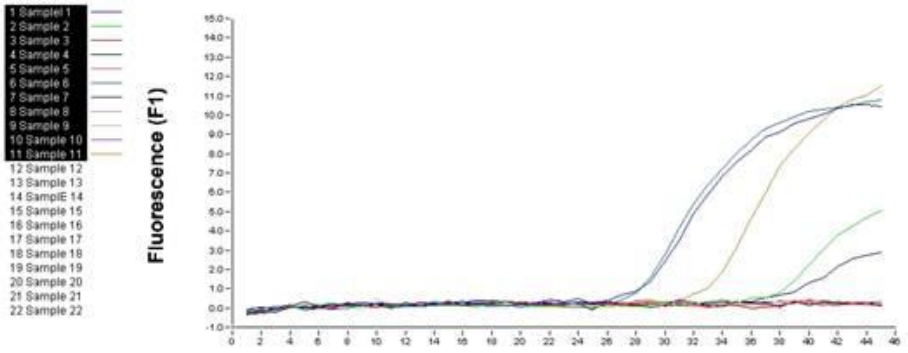
} + 300ul of 10% stool suspension in DDW

Using Nuclisens extraction

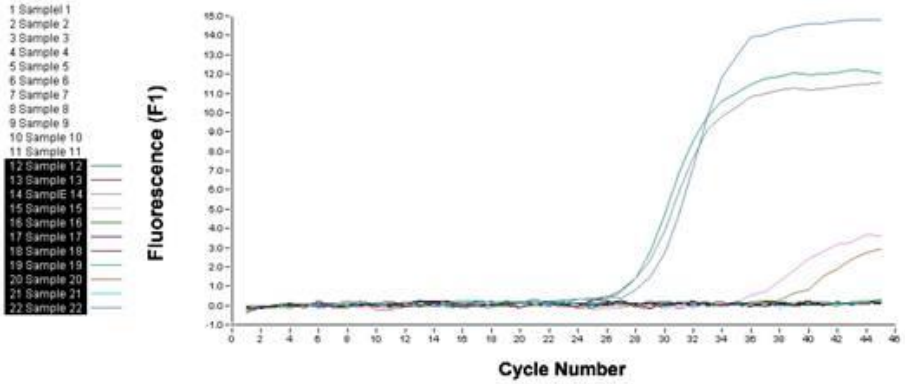
↓

Elute 100 uL

Target	Conc. (CFU/mL)	LOD/dilution		1,000 LOD/dilution	
			~Ct		~Ct
ETEC-LT	1.5×10^8 CFU	1.5×10^4 CFU /D4	35	1.5×10^7 CFU /D1	25
Shi-ipaH	1.5×10^8 CFU	1.5×10^3 CFU /D5		1.5×10^6 CFU /D2	
CR	1.0×10^6oocysts	10 oocysts/D5		10,000 oocysts/D2	



Name	Tube	ID	Ct
Samp1	T1	10^3 LOD_LT	28.14
Samp2	T2	LOD_LT	37.26
Samp3	T3	10^3 LOD_Shig	-
Samp4	T4	LOD_Shig	-
Samp5	T5	NegStool	-
Samp6	T6	10^3 LOD_LT + Inhibitor	27.61
Samp7	T7	LOD_LT + Inhibitor	37.26
Samp8	T8	10^3 LOD_Shig + Inhibitor	-
Samp9	T9	LOD_Shig + Inhibitor	-
Samp10	N	NTC	-
Samp11	P	POS	32.82



Name	Tube	ID	Ct
Samp12	T1	10^3 LOD_LT	-
Samp13	T2	LOD_LT	-
Samp14	T3	10^3 LOD_Shig	27.59
Samp15	T4	LOD_Shig	35.31
Samp16	T5	NegStool	-
Samp17	T6	10^3 LOD_LT + Inhibitor	-
Samp18	T7	LOD_LT + Inhibitor	-
Samp19	T8	10^3 LOD_Shig + Inhibitor	27.32
Samp20	T9	LOD_Shig + Inhibitor	37.94
Samp21	N	NTC	-
Samp22	P	POS	28.8

Positive Extraction Control (PEC), Negative Extraction Control (NEC), Internal Positive Control (IPC) and Comparator Test

Negative Controls

No template control (NTC) - The NTC reaction contains buffer and all of the assay components except nucleic acid. These controls ruled out contamination with target nucleic acid or increased background in the amplification reaction. No template control reactions were manufactured by Idaho Technology, Inc (now BioFire Diagnostics), Salt Lake City, Utah.

Negative sample control

The negative sample control contained non-target nucleic acid. When used to evaluate extraction procedures it contained whole organism to reveal non-specific priming or detection and to indicate that signals were obtained in the absence of target sequences. Negative sample control materials included:

- Patient specimen from a infected individual
- Samples containing a non-target organism

Positive Controls

Positive control for complete assay - The positive control contained well characterized target nucleic acid to control the entire assay process, including DNA extraction, amplification, and detection. It was designed to mimic a patient specimen and to be run as a separate assay, concurrently with patient specimens at a statistically significant frequency. As stated in the proposal, the development and validation of a positive control will require additional funding through follow-on proposal.

Positive control for amplification/detection (PTC) - The PTC for amplification/detection contained purified well characterized target nucleic acid. The PTC was designed to report fluorescence at or near the LoD. The PTC controlled the integrity of the patient sample and the reaction components when negative results were obtained and to indicate that the target is detected if present in the sample. The PTC was manufactured by Idaho Technology, Inc (now BioFire Diagnostics), Salt Lake City, Utah.

Internal Positive Control - Various candidate PCR internal positive controls (IPC) were evaluated for down-selection and testing. The IPC is a non-target nucleic acid sequence that is co-extracted and co-amplified with the target nucleic acid. It will control the integrity of the reagents (polymerase, primers, etc.), PCR instrument function, and the presence of inhibitors in the samples. The human housekeeping gene β-actin was selected for IPC development. As stated in the proposal, the IPC will require additional funding through follow-on proposal.

Comparator Test – gold standard methodology was used as the comparator test (culture) with confirmation testing of amplicon by DNA sequencing. In addition, ETEC LT, ETEC STIa, and

ETEC STIb diagnostic sensitivity test results were compared to a DIG multiplex reaction for the ETEC agents. Shigella diagnostic sensitivity results were compared to serological analyses.

Assay Storage Conditions - A thermal stability study demonstrated that the assays generate equivalent results at several time points throughout the duration of the recommended storage and at both ends of the recommended temperature range. Thermal stability studies were conducted under the GME project. See Results section "Graduate Medical Education Project".

ABI 7900 Transfer

Transfer of JBAIDS formatted assays to the ABI 7900 was successfully completed. Study design is shown in Table 2. Optimized assay formulations and reaction conditions are shown in Table 3. Average Ct Values of Standard Curves from triplicate of 4-folds serial dilutions of ETEC, Shigella, and Cryptosporidium assays are shown in Table 4. Standard curves are shown in Figures 1 -5. Limit of detection estimation derived from standard curve are shown in Table 5. These data include results from both 'JBAIDS ETEC/Shigella' and 'JBAIDS Cryptospordium' projects.

Standard cuve for estimate LOD

Materials and Methods:

1. Isolated colonies for each pathogens were selected and picked from sub culture agar plate (1 loopful)
2. The colonies were suspended in normal saline separately.
3. Suspended colonies were measured at 625 nm and adjusted to 0.5 McFarland (OD_{625nm} 0.088 – 0.133).
4. Nucleic acids extraction was performed using boiling method.

Table 2. OD_{625nm} and Nanodrop measurement of extracted DNA

Strain	OD_{625nm}	Concentration of cell suspension (cell/mL)- 0.5 McFarland
ETEC-STIa	0.106	1.5×10^8
ETEC-STIb	0.091	1.5×10^8
ETEC-LT	0.090	1.5×10^8
Shigella-ipaH	0.088	1.5×10^8
Isolates of Cryptosporidium parvum from Waterborne Inc., using Qiagen extraction kit		
Parasite	NanoDrop measurement	Concentration of undiluted DNA sample
Cryptosporidium-CR	25.5 ηg/µL	10^6 oocysts/µL

PCR amplification and detection

Table 3. The PCR reactions contain the following reagents at specified concentrations:

Reagents	ETEC (STIa, STIb, LT)	Shigella (ipaH)	Cryptosporidium (18S r-RNA)
10X bufferA		1X	
dNTPs (mM)		0.2	
TaqGlod		0.5 U	
Mg^{2+} (mM)	2.5	2.0	3.0
Probe (ηM)	100	100	100
Primer (µM)	0.2	0.2	0.2
Template (µL)		2	
Total Vol. (µL)		20	

Thermo cycler Condition: 95 °C 10 min, 40Cyclers of 95 °C 15 sec and 60 °C 1 min. Template was diluted serially at 4 folds from dilution 1 to dilution 9 (approximately 3.75×10^7 - 5.72×10^2)

Table 4. Average Ct Values of Standard Curves from triplicate of 4-folds serial dilutions of ETEC, Shigella, and CR assays

Strain / Dilution	ETEC-STIa assay		ETEC-STIb assay		ETEC-LT assay		ipaH assay		CR assay	
	Ct Av.	STDEV	Ct Av.	STDEV	Ct Av.	STDEV	Ct Av.	STDEV	Ct Av.	STDEV
D1	21.48	0.431	21.43	0.333	22.34	0.086	19.48	0.154	20.12	0.294
D2	24.58	0.194	23.63	0.332	24.29	0.362	21.29	0.241	22.02	0.244
D3	26.75	0.460	25.69	0.159	26.66	0.220	23.51	0.122	23.77	0.179
D4	28.94	0.448	27.80	0.108	28.75	0.119	25.47	0.196	25.80	0.208
D5	30.71	0.226	29.85	0.150	30.80	0.106	27.58	0.304	28.23	0.212
D6	33.16	0.342	32.35	0.400	32.98	0.393	29.54	0.169	29.98	0.346
D7	35.47	0.216	34.65	0.242	34.92	0.289	31.38	0.100	31.96	0.513
D8	37.54	0.478	37.44	1.560	36.87	1.064	33.71	0.189	34.48	0.616
D9	NA	NA	NA	NA	NA	NA	35.77	1.385	36.76	0.940
Threshold	0.04		0.04		0.04		0.1		0.1	
R^2	0.995		0.989		0.993		0.996		0.997	
Y-Intercept	50.934		52.822		49.455		47.393		39.303	
Slope	-3.699		-3.776		-3.489		-3.486		-3.395	
Figure	1		2		3		4		5	

Av. = Average; STDEV = Standard deviation

Figure 1. Standard curve for ETEC-STIa

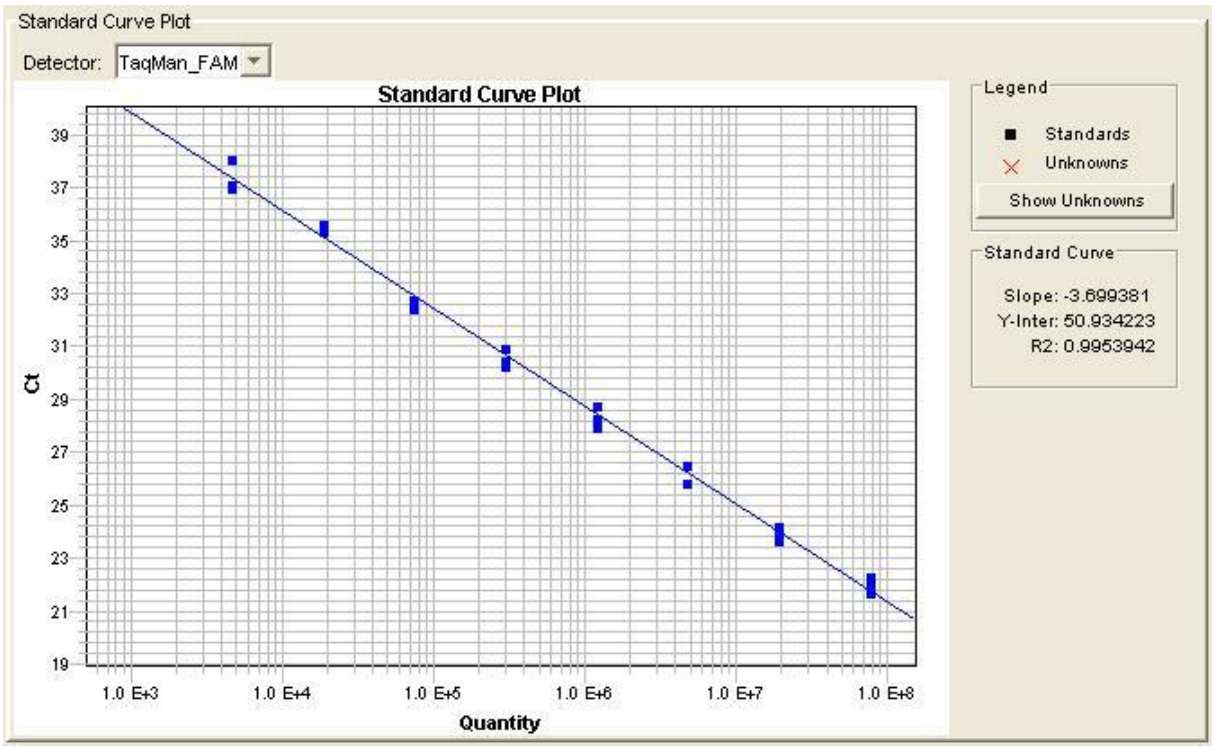


Figure 2. Standard curve for ETEC-STIb

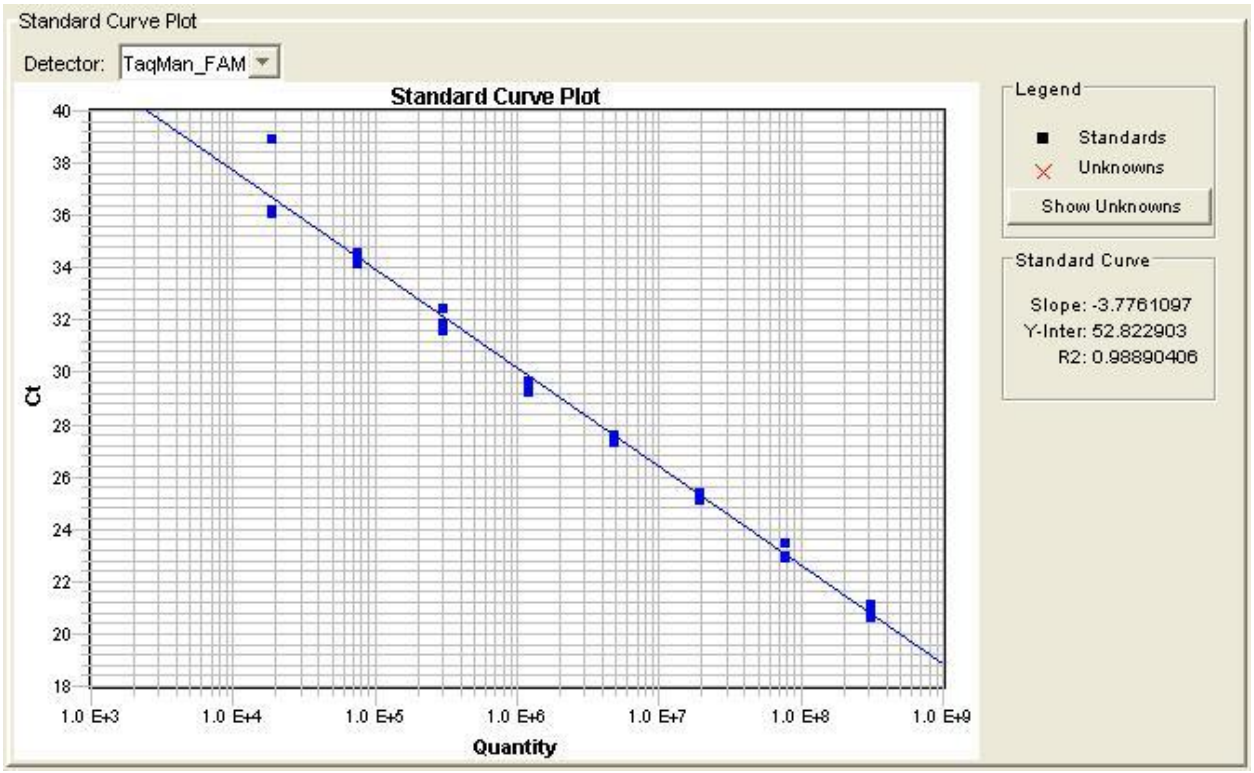


Figure 3. Standard curve for ETEC-LT

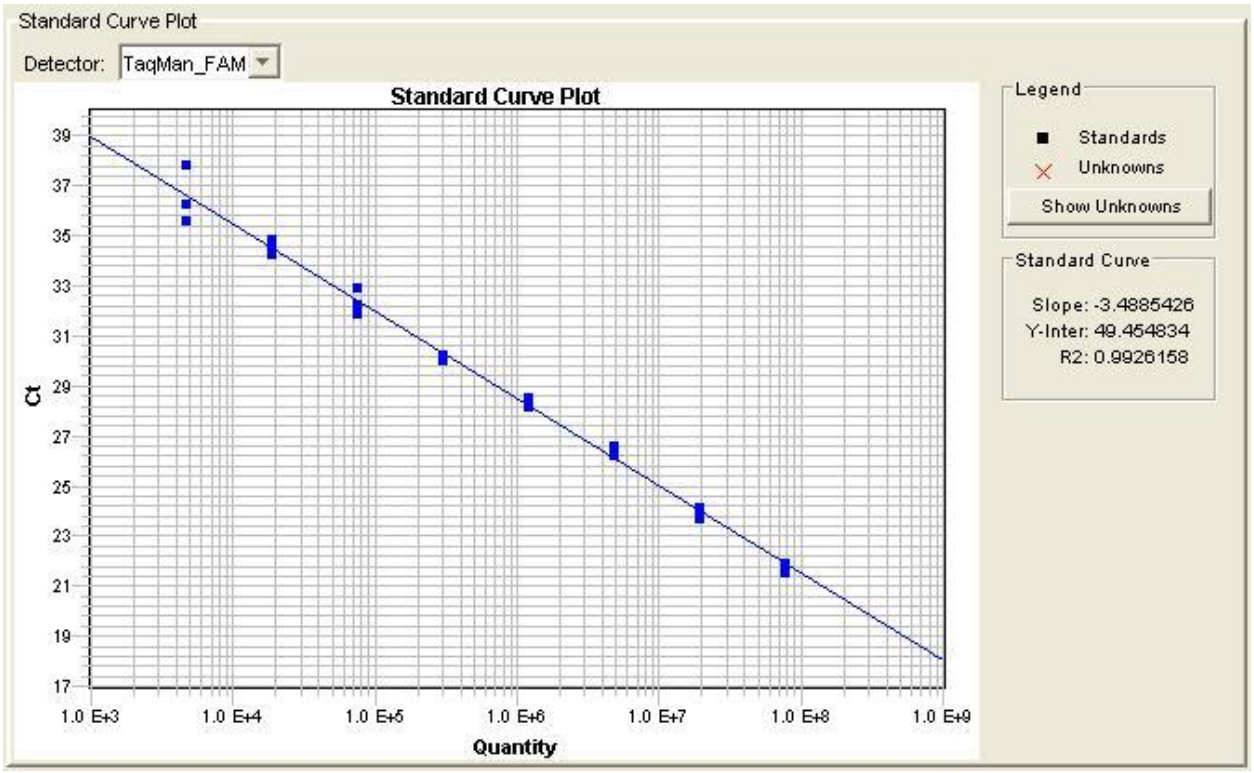


Figure 4. Standard curve for Shigella-ipaH

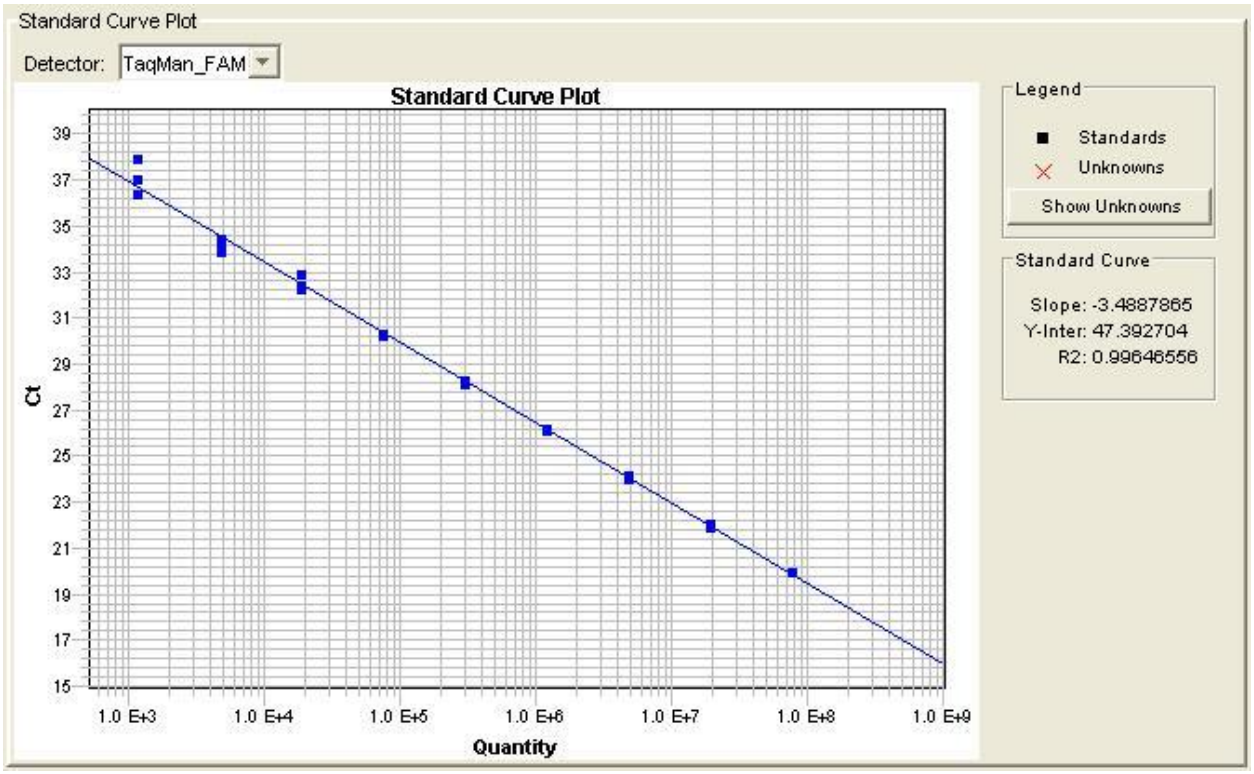


Figure 5. Standard curve for Cryptosporidium

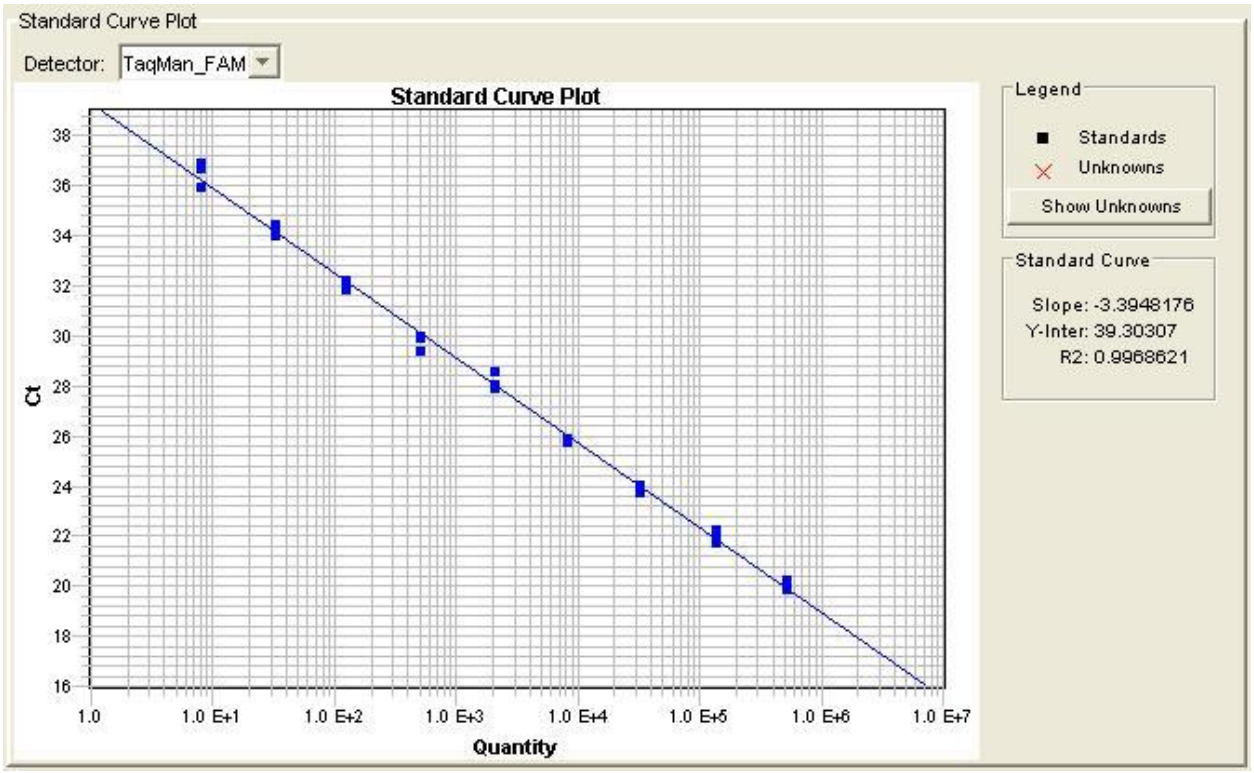


Table 5. LOD estimation derived from standard curve

Assay	Dilution (calculated concentration)*	ABI7900		LOD of RAPID
		Ct average	STDEV**	CFU/mL
ETEC-STIa	D8 (2.29×10^3 CFU/mL)	37.54	0.478	1.5×10^4
ETEC-STIb	D8 (2.29×10^3 CFU/mL)	37.44	1.560	1.5×10^4
ETEC-LT	D8 (2.29×10^3 CFU/mL)	36.87	1.064	1.5×10^4
Shigella-ipaH	D9 (5.72×10^2 CFU)	35.77	1.385	1.5×10^3
Crytosporidium	D9 (3.81 Oocysts/µL)	36.76	0.940	Oocysts/µL 1×10^1

*The last detectable dilution was used to calculate the CFU/mL from starting concentration of $1.5x10^8$ CFU/mL. However, in a LOD experiment, the dilution used for this calculation should be the dilution that shows consistent detectable Ct throughout the experiment which does notnecesarily have to be the last detectable dilution.
**STDEV = Standard deviation

Graduate Medical Education Project

Graduate Medical Education training was conducted during 29 August – 28 September, 2011 at the Enteric Diseases Department, AFRIMS.

GME Resident: Capt Rebekah Piegols, M.D.

GME Mentor: Col Joseph Peter Ray Pelletier, M.D.

Principal Investigator: James C. McAvin and Co-PI: COL Carl Mason, Chief, Department of Enteric Diseases, AFRIMS.

Project results were formatted as an abstract and presented at a medical symposium; Texas Society of Pathologists Symposium, Dallas TX, Jan 13-14 2012 (poster presentation).

Two separate research projects were completed, abstracts prepared and submitted for presentation at a medical symposium;

1. Rebekah Piegols MD, Joseph Pelletier MD, James McAvin. Shigella PCR Taqman Kit Stability Over Time. Texas Society of Pathologists Symposium, Dallas TX, Jan 13-14 2012 (poster presentation).

"During this time of economic restriction, there is an increased pressure on the scientific community to cut costs and stretch research dollars. We performed assay stability testing on the Shigella ipaH PCR reaction assay produced by Idaho Technologies (Salt Lake City, Utah). The original test kits were produced using good manufacturing practices and field tested in Nepal in the spring of 2009. Afterward, these assays were stored at -25°C. In September 2011, we started testing with the same probe/primer designed test assays on the same instrument with similar samples and identical PCR protocol. Our results showed an average critical point (Cp) value of 27.41 (SD 0.48, n=5) for the Shigella positive template control (PTC). The data obtained in Nepal had an average Cp of 27.24 (SD 0.87, n=3). These averages are within a greater than 95% confidence interval. Secondarily, we demonstrated no loss in limit of detection (LOD). Our results indicated detection to the level of $1.5X10^3$ CFU/ml. Reproducibility with 12 samples at the LOD was verified (mean Cp 38.4 with SD 0.88). The results are comparable to the results found in Nepal two years ago. Furthermore, the correlation coefficient of the standard extract was -0.98 to -1.00. In conclusion, these findings validate the stability of the freeze dried primer/probe Taqman reagent mix and could potentially be used to not only facilitate logistics for future research/clinical testing but also decrease costs. Furthermore, in pre-positioning the inventory of these critical detection assays, public health emergency preparedness is enhanced."

Shigella PCR Taqman Kit Stability Over Time

Rebekah Piegols MD, Joseph Pelletier MD, James McAvin

Introduction: During this time of economic restrictions, there is increased pressure on the scientific community to cut costs and stretch research dollars. Here we performed assay stability testing on the Shigella ipaH PCR reaction assay developed by the Dept. of Enteric Diseases, AFRIMS.

Above: Dr. Piegols loads the RAPID

Materials and Methods: The original test kits were produced in a freeze-dried format (Idaho Technology, Inc., Salt Lake City, UT)and field tested in Nepal in the spring of 2009. Afterward, these assays were stored at -25°C. In September 2011, we started testing with the same probe/primer designed test assays on the same instrument with similar samples and identical PCR protocol.

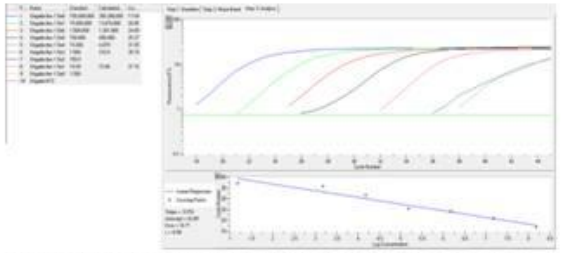


Figure 1: Shigella flexneri limit of detection for two year old reagent stored at -25° C.

Results: Our results showed an average critical point (Cp) value of 27.41 (SD 0.48, n=5) for the Shigella positive template control (PTC). The data obtained in Nepal had an average Cp of 27.24 (SD 0.87, n=3). These averages are within a greater than 95% confidence interval (Table 1). Secondarily, we demonstrated no loss in limit of detection (LOD). Our results indicated detection down to 1.5X10^{3}CFU/ml (Figure 1). Reproducibility with 12 samples at the LOD was verified [(mean Cp 38.4 with SD 0.88) Figure 2]. The results are comparable to the results found in Nepal two years ago. Furthermore, the correlation coefficient of the standard extract was -0.98 to -1.00.

Positive Template Control Values		
	Shigella PTC Nepal 2009	Shigella PTC Thailand 2011
	27.65	27.91
	26.24	27.45
	27.82	27.61
		27.47
		26.62
Average	27.24	27.41
Standard Deviation	0.87	0.48

Table 1: Critical point values for Shigella ipaH PTC in Nepal and Thailand.

Conclusion: In conclusion, these findings validate the stability of the freeze dried primer/probe Taqman reagent mix and could potentially be used to not only facilitate logistics for future research/clinical testing but also decrease costs. Furthermore, in pre-positioning the inventory of these critical detection assays, public health emergency preparedness is enhanced.

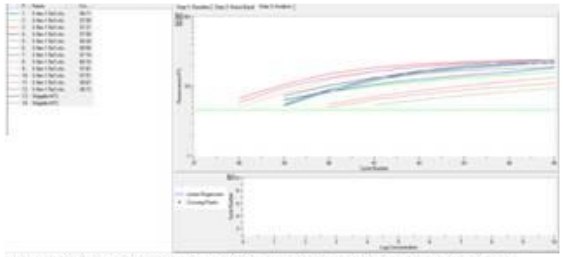


Figure 2: Shigella flexneri 1.5 x 10^3 limit of detection reproducibility; precision test.

References/Acknowledgements:

A big thank you to Dr. Pelletier and Mr. McAvin for their help and patience with me.

Thank you to the Dept. of Enteric Diseases, Armed Forces Institute of Medical Sciences (AFRIMS), Bangkok for the use of their samples and laboratory facilities.

1. Rebekah Piegols MD, Joseph Pelletier MD, James McAvin. Shigella Stool Extraction with Taqman Detection. Texas Society of Pathologists Symposium, Dallas TX, Jan 13-14 2012 (poster presentation).

"We describe here a rapid, field deployable stool nucleic acid extraction process which shows promise for field diagnostic use. A highly modified, streamlined protocol was adapted from a preformatted commercial kit (QIAamp Viral RNA Mini Kit, Qiagen, Valencia, CA). The modified protocol eliminated the need for cold storage or hot water bath. All steps were carried out at ambient temperature using readily available agents. The procedure was performed in approximately one hour for the extraction of ten samples. The extracts were then run on a field-durable, real-time PCR thermocycler the "Ruggedized" Advanced Pathogen Identification Device (RAPID). The limit of detection was $1.5X10^6$ CFU/ml on *Shigella flexneri* extract spiked negative stool. This protocol was further tested using *Shigella sonnei* cultured organisms and *Shigella* positive stool from three different patients. All organisms tested were DNA sequenced or identified via culture methods. There was no cross-reactivity between *Shigella sonnei* and other enteric pathogens including *E. coli* (ETEC) LT, ETEC-ST1a, or ETEC-ST1b. The results were 100% sensitive and 100% specific. These test results demonstrate a rapid and reliable method with potential for field diagnostics in austere environments with further field testing to be completed."

Shigella Stool Extraction with Taqman Detection

Rebekah Piegols MD, Joseph Pelletier MD, James McAvin

Introduction: For at least the last two decades, DNA extraction has been performed on stool specimens, both animal and human. In the deployed or disaster (public health focus) setting, there are limited logistic capabilities with payload limitations. Expanding or extending the use of available pre-packaged materials is set at a premium, especially with the current economy and budget cuts. We propose that the QIAmp Viral RNA mini kit (Qiagen, Valencia, CA) is a viable option to other DNA stool extraction kits. Here we describe a rapid, field deployable stool nucleic acid extraction process which shows promise for field diagnostic use.

Above: Dr. Piegols loads the RAPID. Right: Dr. Pelletier and Mr. McAvin.

Material and Methods: A highly modified, streamlined protocol was adapted from a preformatted commercial kit (QIAamp Viral RNA Mini Kit, Qiagen, Valencia, CA). The modified protocol eliminated the need for cold storage or hot water bath. All steps were carried out at ambient temperature using readily available agents. The procedure was performed in approximately one hour for the extraction of ten samples. The extracts were then run on a field-durable, real-time PCR thermocycler the "Ruggedized" Advanced Pathogen Identification Device (RAPID).

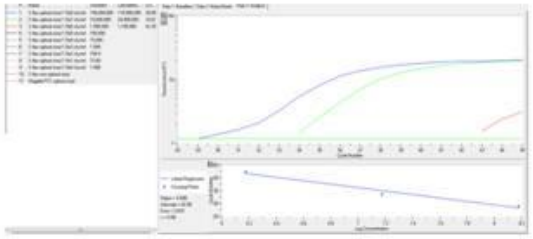


Figure 1: Shigella sonnei limit of detection.

Results: The limit of detection was $1.5X10^6$ CFU/ml on Shigella flexneri extract spiked negative stool (Figure 1). This protocol was further tested using Shigella sonnei cultured organisms and Shigella positive stool from three different patients (Figures 2 and 3). All organisms tested were DNA sequenced or identified via culture methods. There was no cross-reactivity between Shigella sonnei and other closely related enteric pathogens including E. coli (ETEC) LT, ETEC-ST1a, or ETEC-ST1b. The results were 100% sensitive and 100% specific.

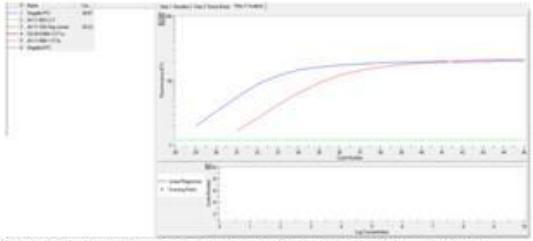


Figure 2: Enteric culture extract cross-reactivity panel of ETEC-LT, ETEC-ST1a, or ETEC-ST1b.

Conclusion/Discussion: Although this was a limited study, these test results demonstrate a rapid and reliable method with potential for field diagnostics in austere environments with further field testing to be completed. The results demonstrated similar detection limits as those found when extracting the DNA from cultured organisms. Detection of Shigella flexneri from DNA extracted from cultures was found to be 1.5×10^3 CFU/ml (by correspondence). Extracting the DNA directly from the patient specimens will save time, energy, manpower and money leading to a more efficient diagnostic procedure. This will not only add to cost savings in medical diagnosis but also decrease the morbidity and potential mortality of patients suffering from infections with these pathogens through a quicker, more specific treatment plan. Further confirmatory tests are in the process to ensure the sensitivity, specificity and ease of extraction protocol developed in this pilot study.

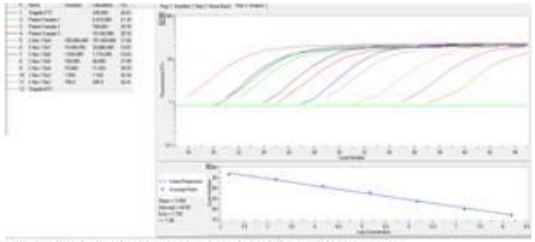


Figure 3: Positive patient samples and limit of detection of spiked stool samples.

References/Acknowledgements:

Abig thank you to Dr. Pelletier and Mr. McAvin for their help and patience with me.

Thank you to the Dept. of Enteric Diseases, Armed Forces Institute of Medical Sciences (AFRIMS), Bangkok for the use of their samples and laboratory facilities.

Conclusion

Work conducted under this study advanced real-time diarrheal disease causative agent diagnostic assays through pre-clinical test phase. Results reported qualify the assays as lead candidates for clinical phase testing. A GME training program was established which provided for scholarly and challenging research opportunity in a real-world environment.

Appendix A

Graduate Medical Education

The investigators conducted the following GME activities: preparation of course materials, development of a research project, preparation of a Research Plan and Training Schedule, integration (and de-integration) of the GME laboratory, reagent and sample preparation, coordination and execution of GME research activities, mentored the resident in proposal writing and results reporting, maintained daily log of resident activities and progress, assured the safety and wellbeing if GME participants. The GME Research Plan and Training Schedule and detailed description of activities are provided below.

Course Summary and Schedule

Research Elective 144

Goals and Objectives: to gain a better understanding of the scientific method and the acquisition of new knowledge through a mentored research experience. The resident will demonstrate ACGME competencies in medical knowledge, practice-based learning and improvement, interpersonal and communication skills, and professionalism.

General objectives are to:

1. Acquaint the resident with a particular area of medical-related research.

2. Teach the resident appropriate research techniques and research design.

3. Assist the resident to complete and write up for publication the results of their research

Specific resident learning objectives for the research project are:

To learn to develop a research question (Medical Knowledge and Practice-Based Learning).

To learn to access, critique, and assimilate the current medical literature pertaining to the research topic (Practice-Based Learning).

To gain an understanding of the scientific method by learning to write an IRB approved research protocol (Practice-Based Learning).

To learn and understand the purpose of informed consent and the regulatory approval process in the setting of research ethics by completing the HIPPA compliance training and obtaining IRB approval for the proposed research project (Professionalism and Systems-Based Practice).

To perform the research and develop the necessary skills required to do this such as laboratory techniques and computer skills (Practice-Based Learning).

To learn and apply the appropriate data analysis and basic biostatistics needed for the project (Practice-Based Learning).

Outcomes assessment: Subjective - A standard competency-based trainee evaluation will be completed at the end of the rotation by the faculty research mentor.

Evaluation

All projects are graded by the Program Director or Associate Program Director using the standard score sheet on the SAUSHEC web site (see appendices A and B). A minimum score of 60 is required to graduate. It is highly recommended that the resident strive for first authorship on a publication in any of the categories listed in the appendix.

Course objectives: the objective of this course is to meet Resident Program requirements in the conduct and completion of a research rotation.

The resident will demonstrate knowledge and proficiency in:

1. IRB protocol and associated documentation preparation and progress reporting.

2. Proposal preparation, funding application preparation and submission, and reporting process.

3. Operation of DoD approved analytic instrumentation (RAPID/JBAIDS) and conduct testing under deployed conditions.

4. The conduct and completion of a research project and results reporting.

5. The preparation of a scholarly abstract and submission to a scientific meeting or symposium.

6. Presentation of results at a scientific symposium, conference, or meeting.

The student will meet or exceed the requirements for completion of Research Elective 144. At the conclusion of the course the student will have prepared a research pre-proposal and statement of work that is suitable for submission for funding. The student will be prepared to submit the associated IRB documentation. The student will have demonstrated the ability to independently conduct and complete a research project and report the results.

Week 1

29 August, 2011

Course Preparation and Travel to Field Site

Monday 29

Literature review and conduct literature search (suggested key words; diarrheal disease, ETEC, Shigella, Cryptosporidia diagnostics, real-time PCR, RAPID/JBAIDS).

Send itinerary and contact information to PI.

Tuesday 30

Review and organize travel file (travel documents, readiness file, GME Training Plan, ETEC/Shigella and Cryptosporidium proposals, and research articles).

Wednesday 31

Pack and prepare for departure. Confirm link-up time/location with travel companions and confirm with PI contact information, arrival time and pick-up location.

Thursday 1

~ 08.00 - Depart US for Field Site (AFRIMS, Bangkok)

Friday 2

23.00 - Arrive Bangkok, transportation to lodging

24.00 - Hotel (Royal View Bangkok)

Sat -Sun - Orientation (local)

Week 2

5 September, 2011

Orientation and Training

Monday 5 (Labor Day Holiday)

0930- 1130 - Course Goals & Objectives, Project Background, and Technology Overview (EDS Dept. Conf. Room, AFRIMS) – established associated course exercises.

GME Goals and Objectives

The student will meet or exceed the requirements for completion of Research Elective 144. At the conclusion of the course the student will have prepared a research pre-proposal and statement of work that is suitable for submission for funding. The student will be prepared to submit the associated IRB documentation. The student will have demonstrated the ability to independently conduct and complete a research project and report the results.

A. Demonstrated knowledge and skill in;

I. Initiative development and project definition.
II. Request For Proposals (RFP) - announcement search.
III. RFP - application process.
IV. Proposal development - statement of work (SOW).

Exercise 1: Essay on the Scientific Method (~ 250 words): COMPLETED.

Exercise 2: Define a project, locate an appropriate funding source, and write a pre-proposal/SOW: COMPLETED.

B. Demonstrated knowledge and skill in;

I. Role and function of Institutional Review Board (IRB)
II. IRB protocol requirements and associated documentation.
III. IRB review process

Exercise 3: Complete CITI and HIPPA compliance training: COMPLETED.

Exercise 4: Essay on IRB function and purpose of informed consent (~ 250 words): COMPLETED.

C. Demonstrated ability to;

I. Independently conduct a sub-component of an ongoing research project.
II. Prepare a scholarly research abstract.
III. Successfully submit and present results at a scientific meeting or symposium.

Exercise 5. Complete a laboratory practical on sample preparation and analyses: COMPLETED (97% concordance using a 30 sample test panel).

Exercise 6. Complete statistical analyses of results: COMPLETED.

Exercise 7: Write a research abstract: COMPLETED.

Exercise 8: Submit abstract and present results: TO BE COMPLETED.

1230-1630 - review of course study materials.

Tuesday 6

0930- 1130 - Overview of Proposal Submission Process: Scientific Method, Proposal Application Process, IRB Submission Process (EDS Dept. Conf. Room, AFRIMS).

1230-1630 – RAPID check-out and review of course study materials.

Wednesday 7

0900-1200 - Proposal Development, IRB Review Process, Molecular Biology Tutorial (EDS Dept. Conf. Room, AFRIMS).

1300-1700 - Proposal Development: proposal topic and documentation preparation (pre-proposal). Review of Molecular Biology Tutorial study materials.

Thursday 8

0900-1200 Laboratory Tour and Orientation (Enteric Diseases Dept, AFRIMS)

1300-1700 GME laboratory set up, RAPID system configuration *(Lecture & Training),* **RAPID system check-out** *(Lecture & Training* - RAPID test run conducted with positive controls reactions (ETEC LT).

Friday 9

0800-1130 Qiagen kit extraction *(Lecture & Training)* - stool extraction protocol and PEC development activities conducted.

1130-1700 Results review, research activity briefing, protocol reviews, and proposal development exercise.

Sat - Sun - Results review, research activity briefing, protocol reviews, and proposal development exercise.

Week 3

12 September, 2011

Training

Monday 12

0800-1200 Sample preparation *(Lecture & Training)* - deployable stool nucleic acid extraction protocol and PEC development activities conducted.

1300-1700 Sample preparation – The student was trained and successfully conducted a limit of detection (LOD) experiment and LOD reproducibility testing using the Shig ipHa RAPID freeze-dried assay and relevant extracts.

Tuesday 13

0800-1200 Nucleic acid preparation - the student was trained and successfully conducted activities toward the development of a deployable stool extraction protocol using the Shig ipHa RAPID freeze-dried assay and relevant samples.

1300-1700 Real time PCR detection - the student conducted PCR analyses of stool extract using the Shig ipHa RAPID freeze-dried assay and relevant samples. Planned and coordinated follow-on development activities for the deployable stool extraction protocol. Continued work on exercises 1, 2, and 4.

Wednesday 14 0800-1700 (1 hour lunch)

Nucleic acid preparation and real time PCR detection - the student conducted an experiment to determine the LOD of Shig ipHa PCR assay using spiked stool samples at 1.5e8 to 1.5e0 cfu/ml concentrations. No fluorescence was reported. The student conducted trouble-shooting and learned that the experiment was inadvertently set-up using ETEC LT template. The PCR was repeated using 1.5e8 cfu/ml concentration and fluorescence reported at the expected Ct. Continued work on exercises 1, 2, and 4.

Thursday 15 0800-1700 (1 hour lunch)

Nucleic acid preparation and real time PCR detection - the student conducted an experiment to determine the LOD of Shig ipHa PCR assay using spiked stool samples at 1.5e8 to 1.5e0 cfu/ml concentrations. Continued work on exercises 1, 2, and 4.

Friday 16 0800-1700 (1 hour lunch)

Results review, follow-on research activity planning and coordination, protocol review and revision, and proposal development exercise. Exercises 1 and 4 completed; essays on the "Scientific Method" and "IRB Function and Purpose of Informed Consent.

Week 4

19 September, 2011

Training and Data Collection

Monday 19 0800-1700 (1 hour lunch)

Real time PCR detection (Proficiency Evaluation) - preparation of a 30 sample test panel consisting of well characterized *Shigella,* ETEC LT, and ETEC ST1b nucleic acid extracts from archived patient samples. The student independently prepared master mix using the respective detection assays and conducted PCR in a blind format. The student's results were 97% concordant with the sample key. A single Shigella sample reported weak fluorescence (Ct = 40) by ETEC LT PCR analysis. The student repeated testing of the discordant sample with six additional shigellosis sample extracts and found all samples negative by ETEC LT PCR analyses.

Tuesday 20 0800-1700 (1 hour lunch)

Sample Preparation and Real time PCR detection *(*Proficiency Evaluation*)* - the student prepared *Shigella flexneri,* ETEC LT, and ETEC ST1a and ETEC ST1b spiked stool samples using isolates. Nucleic acid extract was prepared and PCR conducted using the Shig ipHa PCR assay. Results were 100% concordant with identification by culture, the *S. flexneri* spiked sample reported fluorescence and all other spiked stool samples were negative by Shig ipHa PCR analyses (results are described in detail in the associated abstract). The student designed an experiment to evaluate the performance of the deployable sample preparation protocol successfully quantified limit of detection using linear regression analyses of a standard curve comprised of eight logs of known concentration (results are described in detail in the associated abstract).

Wednesday 21 0800-1700 (1 hour lunch)

Conduct research for abstract, Pre-proposal preparation and completion of IRB essay.

Thursday 22

0800-1200

Sample preparation and Real time PCR detection: Last experiment with patient samples previously proven positive for Shigella via cultures. Extraction performed on positive patient samples. Real time PCR performed using Stool extracts positive and negative PTC and with standard curves. Able to detect to 1.5 E2 templates and quantitative copies of Shig ipaH in the stool sample.

1300-1700

Results review. Continue research for abstract. Finalize proposal. Finalize essay on IRB. Continue writing abstracts. Symposium planning.

Friday 23

0800-1700 Results review. Abstract preparation and pre-proposal/SOW development.

Week 5

26 September, 2011

Results Review, Data Archiving, and Re-deployment

Monday 26

0800-1200 Results review, abstract preparation, and complete proposal development exercise.

1300-1700 Organize and archive research results and symposium planning.

Tuesday 27

0800 -1700 Pack and prepare for departure (hotel check-out).

Wednesday 28

- Transportation to airport, Depart Bangkok (~ 0800) / Arrive U.S. (~ 2000)

Appendix B

Pathology Resident Research Electives (from Dept. of Pathology Handbook)

Research Elective 144

Research Elective Rotation

Course Director:

All activities will be supervised by;

Director of Resident Research: Dr. Wade Aldous

Residency Program Director and/or Associate Director: Drs. Daniel Cruser and Dale Selby.

Resident Research Mentor: Dr. Peter Pelletier

Rotation period: Elective rotation, offered for 1 month.

General organization: Participation in research during residency training
Can provide valuable experience regardless of ultimate career goals and is a SAUSHEC graduation requirement.

As such, the Department of Pathology offers a Research Elective to provide protected time for participation in a research project, as well as support in all phases of conception and implementation of projects. Using elective time is not required for completion of the graduation research requirement, however, and residents may choose to do research without taking this elective.

Pathology residents may receive elective credit for up to 4 months of research time, which need not be contiguous, during their PGY-2 thru PGY-4 years. It is anticipated that most research projects will take place over the course of several months to four years, with protected elective time allocated for periods of intensive work such as background literature reviews, data collection, or data analysis.

Rotation Goals and Objectives: The goal of the resident research program is for the resident physician to gain a better understanding of the scientific method and the acquisition of new knowledge through a mentored research experience. The resident will demonstrate ACGME competencies in medical knowledge, practice-based learning and improvement, interpersonal and communication skills, and professionalism.

Reference: AGGME

General objectives of the Pathology Research Elective are to:

1. Acquaint the resident with a particular area of pathology-related research.

2. Teach the resident appropriate research techniques and research design.

3. Assist the resident to complete and write up for publication the results of their research Specific resident learning objectives for the research project are:

To learn to develop a research question (Medical Knowledge and Practice-Based Learning).

To learn to access, critique, and assimilate the current medical literature pertaining to the research topic (Practice-Based Learning).

To gain an understanding of the scientific method by learning to write an IRB approved research protocol (Practice-Based Learning).

To learn and understand the purpose of informed consent and the regulatory approval process in the setting of research ethics by completing the HIPPA compliance training and obtaining IRB approval for the proposed research project (Professionalism and Systems-Based Practice).

To perform the research and develop the necessary skills required to do this such as laboratory techniques and computer skills (Practice-Based Learning).

To learn and apply the appropriate data analysis and basic biostatistics needed for the project (Practice-Based Learning).

Research Elective 145

To demonstrate communication skills by presenting research results to program directors and fellow residents and/or presenting results at a national meeting and/or writing a paper for publication in medical journals (Interpersonal and Communication Skills).

Resident Duties and Responsibilities:

To receive elective credit for research, the resident must complete the following minimum requirements:

. Identify a faculty research mentor and proposed project.

. Submit a brief (1-2 page) summary of a proposed research project and a research plan with study design and timeline (which may consist of the IRB protocol) to be approved by Residency Program Director or Associate Director and the research mentor.

. Complete the Collaborative Institutional Training Initiative (CITI training) on line research training module.

. Obtain regulatory approval for the project, as appropriate. In most cases this will include writing and submitting a protocol to the IRB.

. Present findings to fellow residents and program directors or at a national meeting in the form of a poster or as a publication in a medical journal.

. Submit a final product to the program directors. This may be an abstract, a poster presentation, the draft of a paper, or a publication.

. Attend all regularly-scheduled academic conferences, other military duties, and conferences as assigned.

. Obtain prior approval for time spent away from the primary training sites (BAMC and WHMC).

Outcomes assessment: Subjective - A standard competency-based trainee evaluation will be completed at the end of the rotation by the faculty research mentor.

Additional Information

Note that use of the term "research" may be interpreted broadly to encompass a range of scholarly pursuits. Dr. Aldous and the program directors are available to help residents identify potential research mentors and scholarly projects. Residents also have access through the medical center to many resources ranging from computer classes, seminars on clinical investigation, and statistics help.

The requirements listed above are only minimal requirements. It is hoped that participating residents will also take advantage of the research elective opportunity to develop new skills, present at national meetings, and write up the results of their research for journal publication.

SAUSHEC Graduation Paper Requirement

Research Opportunities

1. Several pathology staff have ongoing projects in which you can participate or start. These are usually presented at the Research Committee Meeting.

2. Cancer Therapy and Research Center (CTRC) collaboration. The CTRC has many opportunities for residents to participate in original research. Most projects involve benchtop work using molecular techniques. These projects are designed to result in a publication.

3. Elective research month. You can use an elective month or more for research. See the handbook for details.

Timeline

1. You should have a project by the end of your second year.

2. Plan to submit your manuscript by the middle of your senior year, at the very latest.

3. Warning!!!! The SAUSHEC Graduate Medical Education Committee (GMEC) starts to review resident compliance with the SAUSHEC research requirement by early Fall of your senior year. Program Directors are required to present non-compliant residents by name to the GMEC. Continued non-compliance will result in adverse action. It is SAUSHEC policy that if you do not complete the research requirement you will not receive your graduation certificate.

Evaluation

All projects are graded by the Program Director or Associate Program
Director using the standard score sheet on the SAUSHEC web site (see below).
A minimum score of 60 is required in order to graduate. In general, if you are first author on a publication in any of the categories listed above, you will likely pass.

SAUSHEC Graduation Paper Requirement

SAUSHEC GRADUATION PAPER

SCORE SHEET

Total Percentage Points: 100%

A score under 60% is considered unsatisfactory.

1. Originality of project (10 pts)

Score
1 2 3 4 5 6 7 8 9 10 _____

Comments:

2. Review/Discussion of Literature /Quality of Introduction (10 pts)

1 2 3 4 5 6 7 8 9 10 _____

Comments:

3. Design of Clinical or Animal Research/Case Report/Education project/
Chart or Subject Review (10 pts)

1 2 3 4 5 6 7 8 9 10 _____

Comments:

4. Data Analysis/Results/Graphics (20 pts)

1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20 _____

Comments:

5. Quality of Discussion (20 pts)

1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20 _____

Comments:

6. Effort required to design and execute Study/Project (10 pts)

1 2 3 4 5 6 7 8 9 10 _____

Comments:

7. Scientific/Academic merit/significance of project (10 pts)

1 2 3 4 5 6 7 8 9 10 _____

Comments:

8. Style (Sentence structure/grammar/clarity of thought) (10 pts)

1 2 3 4 5 6 7 8 9 10 _____

Comments:

TOTAL = _____

PROGRAM DIRECTOR:_____

Signature Date

Appendix C

Through earlier AF/SGR funded projects we developed highly sensitive and specific, dual-fluorogenic, hydrolysis probe (TaqMan), RAPID/JBAIDS PCR assays for the detection of enterotoxigenic *Escherichia coli* (ETEC) toxin genes, Heat Stable Ia (STIa), Heat Stable Ib (STIb), and Heat Labile (LT) and *Shigella*/enteroinvasive ***E. coli*** (EIEC) species. Our results indicated that the ETEC and *Shigella* assays are more sensitive than the current gold standard methodology.

The ETEC assay LOD for STIa, STIb, and LT were established at ≤ 1000 fg (≤ 100 genomic equivalent) for each assay (Tables C1 and C2). Typical RAPID run results are shown in Figure C1. Assay *in vitro* sensitivity was 100% and specificity 100% concordant with well characterized *E. coli* reference strains, genetic near neighbors, and broad cross-reactivity panel to include human DNA (Tables C3 and C4). In field-based, blind testing using a panel of fresh stool samples (n=118) STIa, STIb and LT assay sensitivity test results were all 100% concordant and specificity test results were STIa (92.4%), STIb (92.6%), and LT (79.6%) concordant with DNA sequencing results (14).

Table C1. Results linear regression analyses of the ETEC assays using triplicate dilution of positive control template that spanned six logs of concentration

Strain / Dynamic ranges	ETEC-STIa	ETEC-STIb	ETEC-LT
R^2	1	1	1
Slope	-3.515	-3.357	-3.438
Intercept	47.83	47.33	47.87
Error	0.0428	0.0793	0.0283

Based on the standard curves, the 4th dilution of all known ETEC strains (equivalent to $\underline{1.5\times10^4}$ cell/mL) was selected to represent the assays LOD. Those cell concentrations were used to perform downstream reproducibility experiments. Note: R^2 of unity is based on the robustness of linearity achieved by the PCR. For optimized reactions that have met validation criteria, a "Best Fit" algorithm is utilized to automatically calculate correlation at an assumed value of unity. Assay sensitivity and specificity are assumed to be 100% for the calculation of infection rate (16, 17).

Figure C1. Assay reproducibility of ETEC-STIa on RAPID

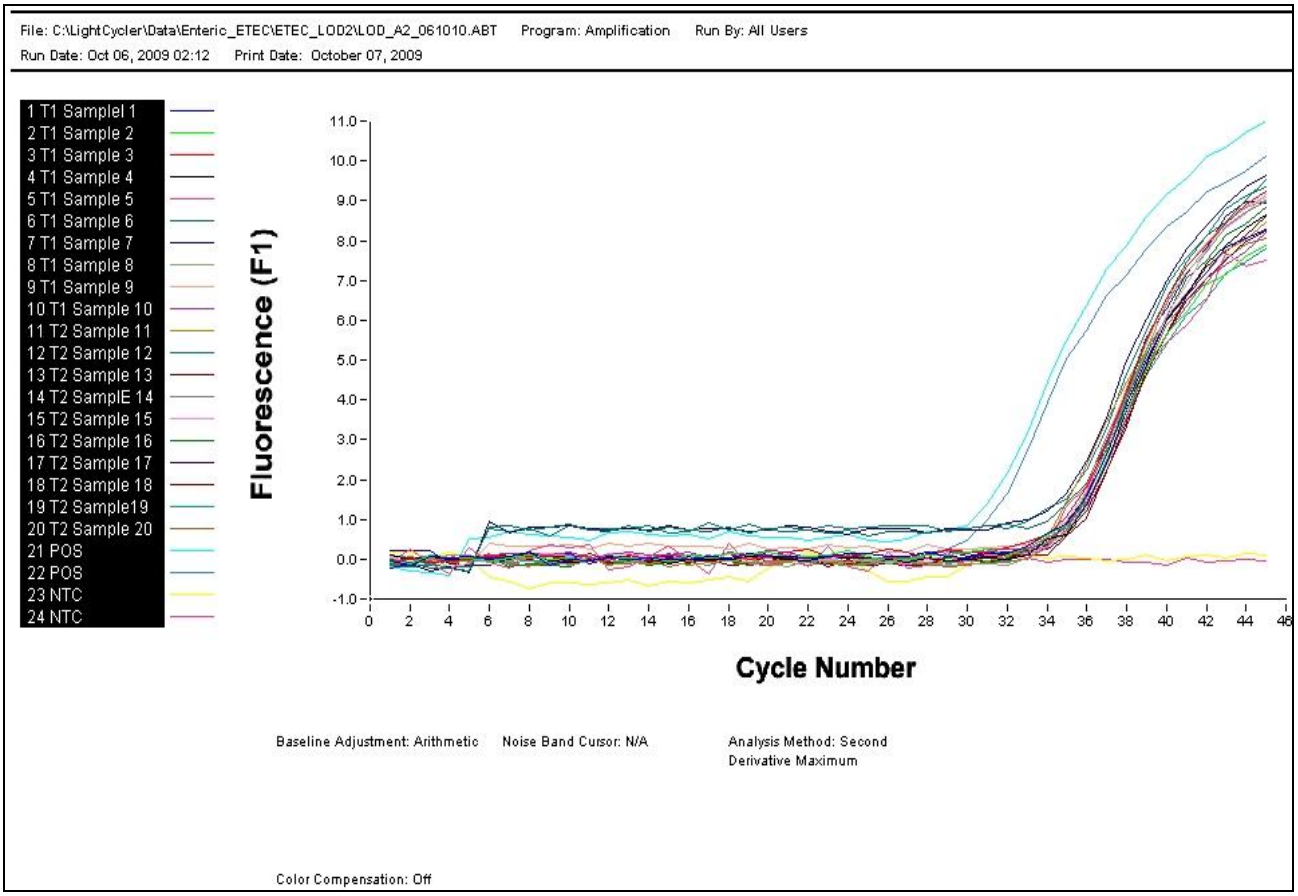


Table C2. Results of assay reproducibility of the LOD determination by performing a total of 60 replicates at three different runs (20 replicates each time) by two different individuals.

Assays / Run	ETEC-STIa assay		ETEC-STIb assay		ETEC-LT assay	
	Mean of Ct	SD of Ct	Mean of Ct	SD of Ct	Mean of Ct	SD of Ct
1	34.07	0.49	33.66	0.77	34.94	0.59
2	34.63	0.37	34.88	0.29	34.56	0.33
3	35.94	0.35	34.07	0.69	34.93	0.43
Total replicates	Pass (60/60)		Pass (60/60)		Pass (60/60)	

Table C3. Determination of assay cross-reactivity with other closely- and distantly-related organisms (exclusivity) at 1000LOD

Sample ID	*Species/Strain*	*Assay*		
		STIa	*STIb*	*LT*
AF-ETEC929	*ETEC-STIa*		*Negative*	*Negative*
AF-ETEC727	*ETEC-STIa*		*Negative*	*Negative*
AF-ETEC877	*ETEC-STIb*	*Negative*		*Negative*
AF-ETEC771	*ETEC-STIb*	*Negative*		*Negative*
AF-ETEC966	*ETEC-LT*	*Negative*	*Negative*	
AF-ETEC083	*ETEC-LT*	*Negative*	*Negative*	
ATCC25931	*Shigella sonnei*	*Negative*	*Negative*	*Negative*
ATCC25922	*Escherichia coli*	*Negative*	*Negative*	*Negative*
ATCC70819	*Campylobacter jejuni*	*Negative*	*Negative*	*Negative*
AF-SAL0085	*Salmonella gr.E4*	*Negative*	*Negative*	*Negative*
AF-SAL445	*Salmonella paratyphi A*	*Negative*	*Negative*	*Negative*

Table C4: List of non-ETEC strains (n=30) used for evaluation of cross-reactivity

Pathogen	***No. of sample***	***TaqMan PCR Interpretation***
Acinetobacter Calcoaceticus	*1*	*Negative*
Arcobacter butzleri	*1*	*Negative*
Campylobacter spp.	*7*	*Negative*
Citrobacter freundii	*1*	*Negative*
EHEC	*1*	*Negative*
Enterobacter aerogenes	*1*	*Negative*
Enterobacter cloacae	*1*	*Negative*
Enteroinvasive E.coli (EIEC)	*1*	*Negative*
Escherichia coli	*1*	*Negative*
K.pneumoniae	*1*	*Negative*
P.aeruginosa	*1*	*Negative*
Proteus hauseri	*1*	*Negative*
Salmonella spp.	*2*	*Negative*
Shigella spp.	*2*	*Negative*
Staphylococcus spp.	*4*	*Negative*
Vibrio spp.	*4*	*Negative*
Total	*30*	

The *Shigella*/EIEC-ipaH assay limit of detection (LOD) was established at < 100 fg (< 10 genomic equivalent) [Tables C5, C6 and Figure C2,C3]. Assay *in vitro* sensitivity was 100% and specificity 100% concordant with well characterized *Shigella* reference strains, near genetic near neighbors, and broad cross-reactivity panel to include human DNA (Tables C4 and C7). In field-based, blind testing with a panel of fresh stool samples (n=118) assay sensitivity test results

were 100% concordant and specificity test results were 81.6% concordant with DNA sequencing results (15).

TableC5. Determination of the assay reproducibility at the LOD by performing a total of 60 replicates at three different runs (20 replicates each time) by two different individuals.

Assay / Run	ipaH assay		
	Mean of Ct	SD of Ct	Date/name
1	36.033	0.650	14Oct09/Sasikorn & Pimmnapar
2	36.326	0.648	15Oct09/Sasikorn & Pimmnapar
3	36.316	0.478	16Oct09/Sasikorn & Pimmnapar
Total replicates	Pass (60/60)		

Figure C2. Standard curve of Shigella/EIEC-ipaH assay on RAPID platform

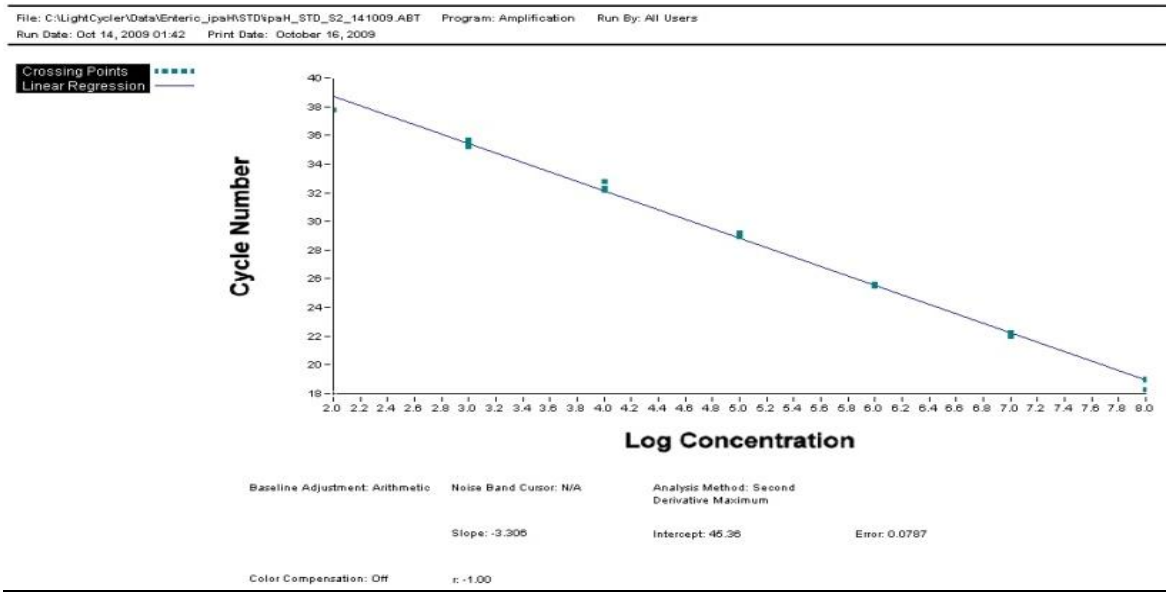


Table C6. Dynamic ranges of the ipaH assays using triplicate dilution of the positive control materials that span at least five orders of magnitude.

Strain / Dynamic range	Shigella
R^2	1
Slope	-3.306
Intercept	45.36
Error	0.0787

Based on linearity evaluation standard curves, the 5th dilution of known *Shigella* strain (equivalent to 1.5×10^3 cell/mL) was selected to represent the assays LOD. Those cell concentrations were used to perform downstream reproducibility experiments. Note: R^2 of unity is based on the robustness of linearity achieved by the PCR. For optimized reactions that have met validation criteria, a "Best Fit" algorithm is utilized to automatically calculate correlation at an assumed value of unity. Assay sensitivity and specificity are assumed to be 100% for the calculation of infection rates.

Figure C3. Assay reproducibility of *Shigella*/EIEC-ipaH assay on RAPID platform

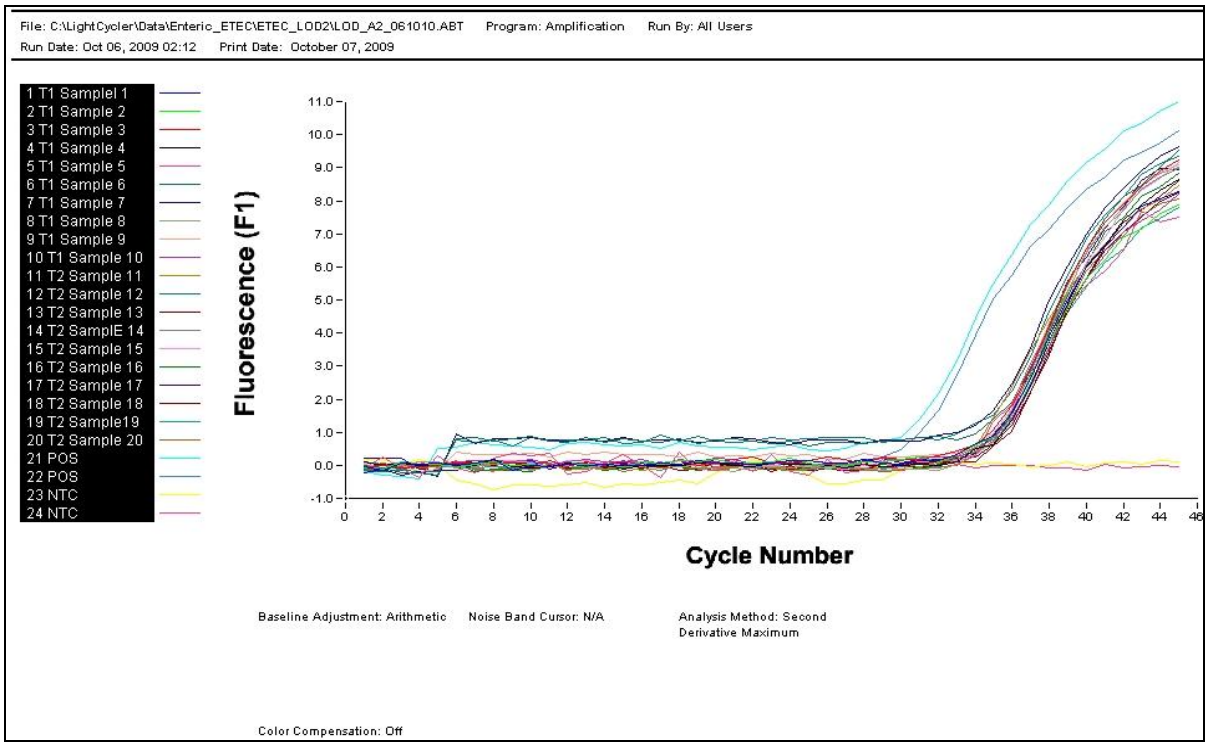


Table C7. IpaH assay exclusivity determination at 1000 LOD on RAPID platform.

Strain	Pathogen	ipaH Assay
AF-ETEC929	ETEC-STIa	Negative
AF-ETEC727	ETEC-STIa	Negative
AF-ETEC877	ETEC-STIb	Negative
AF-ETEC771	ETEC-STIb	Negative
AF-ETEC966	ETEC-LT	Negative
AF-ETEC083	ETEC-LT	Negative
ATCC25922	*Escherichia coli*	Negative
ATCC70819	*Campylobacter jejuni*	Negative
AF-SAL0085	*Salmonella* gr. E4	Negative
AF-SAL445	*Salmonella paratyphi* A	Negative